RESEARCH NOTES

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Isolation of Viable *Toxoplasma gondii* From Naturally Infected Aborted Bovine Fetuses

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ABSTRACT: *Neospora caninum* and *Toxoplasma gondii* are related parasites. The former is a common cause of abortion in dairy cattle. The latter has not been conclusively demonstrated in bovine fetuses. During the course of attempts to isolate *N. caninum* from aborted fetuses, *T. gondii* was isolated from 2 aborted fetuses, 1 from Portugal and 1 from the United States. Both isolates were made by bioassay of fetal brains in mice. The fetus from Portugal was about 5 mo in gestational age, and the fetus from the United States was a full-term stillborn.

Abortion is a major economic problem in the cattle industry worldwide. The diagnosis of abortion is difficult because of multiple etiologies and the advanced stage of autolysis in the many fetuses submitted for diagnosis. Even in well-equipped diagnostic laboratories, the cause of abortion is not determined in about half the fetuses (Anderson et al., 1991). Until the discovery of *Neospora caninum* as a major cause of bovine abortions in 1989 (reviewed in Dubey and Lindsay, 1996), protozoans were considered a rare occurrence in bovine fetuses. Although *Toxoplasma gondii* is a major cause of abortions in sheep and goats (Dubey and Beattie, 1988), a review of worldwide reports indicated that *T. gondii* is not an important cause of abortion in cattle (Dubey, 1986).

Neospora caninum is morphologically similar to *T. gondii*, so much so that their tachyzoties are indistinguishable by light microscopy (Dubey and Lindsay, 1996). Although *N. caninum* has been found in encephalitic lesions in 20–45% of aborted fetuses, attempts to recover viable *N. caninum* have been largely unsuccessful probably because most *N. caninum* die along with the host tissue. In 1 study, *N. caninum* was isolated in only 2 of the 49 histologically confirmed infected fetuses (Conrad et al., 1993). Unlike *N. caninum*, *T. gondii* is easily isolated, even from autolysed tissues (J. P. Dubey, pers. obs.). Therefore, there are only a few isolates of *N. caninum* from bovine fetuses, and the search for new isolates continues (Dubey, 1999). Furthermore, each new *N. caninum*–like isolate must be distinguished from *T. gondii*. Apart from anecdotal evidence, viable *T. gondii* has not been isolated conclusively from naturally aborted bovine fetuses, and attempts to produce transplacental toxoplasmosis in cows fed *T. gondii* oocysts have been largely unsuccessful (Stalheim et al., 1980; Dubey, 1983).

Recently, Gottstein et al. (1998) detected *T. gondii* DNA in 5% of the aborted bovine fetuses in Switzerland. They examined 83 bovine fetuses for the presence of *N. caninum* and *T. gondii* by several diagnostic procedures. They detected *N. caninum* DNA in 24 and *T. gondii* DNA in 4 of the fetuses; in 1 fetus, DNA for both parasites was detected. In addition, they found antibodies to *T. gondii* in 2 dead calves, suggesting transplacental infection. Neither *N. caninum* nor *T. gondii* was found in 54 of the cases. Neither parasite was cultivated in vitro from tissues of 27 of the 83 fetuses that were suitable for in vitro studies. In the most recent study from the same laboratory, *N. caninum* DNA was found in 50 (21%) and *T. gondii* DNA in 1 (<1%) of the 242 aborted fetuses tested (Sager et al., 2001).

Ellis (1998) from Australia also found *T. gondii* DNA in 2 and *N. caninum* DNA in 16 of 40 fetuses with histologically confirmed encephalitis. Therefore, there is a need for reassessing the prevalence of transplacental *T. gondii* infection in cattle. The objective of the present report is to document the isolation of viable *T. gondii* in 2 aborted bovine fetuses, 1 from the United States and 1 from Portugal. Because of the different methodologies used, each isolation is reported separately.

Case 1: A Holstein dairy cow from the state of Washington aborted full-term stillborn twins in October 1992. The fetuses were necropsied by 1 of us (M.W.E.). Brain and thoracic fluids from both fetuses and the serum of their dam were submitted to the USDA's Beltsville Laboratory in an attempt to isolate *N. caninum*. One of these fetuses had severe ascites and short arthrogryposed legs (fetus A).

Brains from each fetus were homogenized in phosphate-buffered saline (PBS) in a blender, washed twice by centrifugation, and the sediment suspended in antibiotic (1,000 units of penicillin and 100 μg of streptomycin/ml of PBS). An aliquot of this homogenate was inoculated into 5 Swiss Webster (SW) mice (females, 20–25 g, Taconic Farms, Germantown, New York). The mice had been inoculated intramuscularly with 4 mg of methyl prednisolone acetate (UpJohn, Kalamazoo, Michigan) to increase susceptibility to *N. caninum* (Lindsay and Dubey, 1989b). Aliquots were also inoculated into Vero cells in culture (Lindsay and Dubey, 1989c).

The mice inoculated with bovine brains remained healthy and were bled 167 days later to exclude *T. gondii* infection. A 1:50 dilution of mouse sera was examined for antibodies to *T. gondii* by the modified agglutination test (MAT) as described (Dubey and Desmonts, 1987). Antibodies to *T. gondii* were found in 1 of the 5 mice inoculated with brain of fetus B and in none of the 5 mice inoculated with tissues of fetus A. The mice were killed 18 days later (185 days after brain inoculation), and their brains were examined microscopically for protozoan tissue cysts; despite intensive searching, only thin-walled *T. gondii* tissue cysts were found in the mouse with *T. gondii* antibodies. The brain of the mouse with tissue cysts was homogenized in PBS and inoculated subcutaneously into another 5 SW mice. All 5 mice inoculated with the bovine isolate of *T. gondii* developed antibodies to *T. gondii* in the MAT, and tissue cysts were found in the brains of all 5 mice when they were killed 84 days later. A parasite-free laboratory-raised cat (Dubey, 1995) fed tissue cysts from this isolate shed *T. gondii* oocysts. Protozoans were not isolated in cell culture inoculated with fetal brains.

Antibodies to *T. gondii* were not detected in a 1:25 dilution of thoracic fluids of the 2 bovine fetuses. The dam of the fetuses had a MAT *T. gondii* titer of 1:100; it was culled and sent to slaughter before the results of *T. gondii* testing were known. Examination of histopathology in the fetuses was not done.

Case 2: A 5-mo-old fetus, aborted in January 2002 by a 32-mo-old Holstein cow from Azores Island, Portugal, was processed for *N. caninum* isolation. For this, half the brain was homogenized in 2% (v/w) PBS–trypsin (Difco, Detroit, Michigan) and incubated in a shaker bath at 37 C for 1 hr. This brain suspension was washed 3 times with 50 ml of PBS by centrifugation at 1,062 *g* for 10 min. The pellet was resuspended in 4 ml of PBS, and approximately 1 ml of the suspension was inoculated intraperitoneally (i.p.) into 4 SW mice previously given 10 mg/ml dexamethasone (Kela Laboratória, Hoogstrated, Belgium) in drinking water ad libitum for 10 days to increase susceptibility to *N. caninum* (Romand et al., 1998). The mice were killed 4 days later, and their peritoneal cavity was washed with 5 ml of PBS. The peritoneal wash was mixed with 2 ml of mouse sarcoma cells Tg 180 (2×10^5 cells/μl) (Desmonts and Remington, 1980). The mixture was then centrifuged for 10 min at 1,062 *g*. The pellet was resuspended in 4 ml of PBS, and approximately 1 ml of this suspension was inoculated i.p. into 4 dexamethasone-treated mice. Four days later these mice were killed, and their peritoneum was washed with 5 ml of PBS. The washing was then incubated with 1% PBS–trypsin at 37 C on a shaker. The mixture was centrifuged 3 times with PBS at 1,062 *g* for 10 min. Parasites in the pellet were reacted with antibodies to *N. caninum* and *T. gondii* using an indirect fluorescent antibody test (IFAT). The IFAT was performed in 12 circles of immunofluorescence slides prepared at the Porto laboratory. For this, parasites from the pellet were placed in each circle and dried at 37 C. After drying, the slides were fixed with 50% meth-

anol for 30 min at 22 C. After fixation, 50 μl of anti–*T. gondii* or anti–*N. caninum* rabbit antiserum (Lindsay and Dubey, 1989a) diluted 1: 5,000 in PBS was placed in each circle. The slides were then incubated at 37 C for 1 hr in a humidified atmosphere. After incubation the slides were washed 3 times in PBS for 30 min. Finally, 50 μl of a goat anti-rabbit fluorescein isothiocyanate–conjugated IgG antibody (Sigma Chemical Co., St. Louis, Missouri) diluted 1:160 in PBS with 0.05% of Evans Blue (George T. Gurr, London, U.K.) was added to each circle in the slides. The slides were then incubated at 37 C for 30 min in a humidified atmosphere and washed 3 times in PBS for 10 min. Immediately after washing, the slides were observed through an epifluorescence microscope (Nikon Optiphot, Tokyo, Japan) at ×200 and ×400 magnifications, using a B-2 A (450–520 nm) filter. Tachyzoites of the RH strain of *T. gondii* were used as the positive control. For the negative control the same parasites were used but incubated with anti–*N. caninum* rabbit antiserum (Lindsay and Dubey, 1989b).

For in vitro propagation of the parasites, part of the remaining pellet was resuspended in minimum essential medium (GIBCO; Paisley, Scotland) with 10% fetal bovine serum (GIBCO) and 2% penicillin–streptomycin (GIBCO). This suspension of parasites was inoculated into a monolayer of Vero cells kept in T25 plates (Nalge Nunc Internacional, Roskilde, Denmark) and incubated in an atmosphere of 5% CO_2 at 37 C.

For in vivo propagation, part of the pellet was resuspended in sterilized PBS at a concentration of 1,000–5,000 tachyzoites per milliliter, and 0.2 ml of this suspension was inoculated in nonimmunodepressed and immunodepressed SW mice.

Tachyzoites were first seen microscopically in the peritoneal exudate of mice coinfected with mouse sarcoma cells and the peritoneal exudate from mice inoculated with the brain homogenate. Microscopical observation of the Giemsa-stained slides showed numerous *T. gondii*–like tachyzoites. These tachyzoites reacted with anti–*T. gondii* antibodies but not with *N. caninum* antibodies in the IFAT. The isolate of *T. gondii* in the present study has been successfully maintained in the Vero cell culture and mice. The fetus was not examined for histopathology.

It is clear from the results presented in this paper that *T. gondii* can be transplacentally transmitted in cattle, but it is probably a rare occurrence. Whether *T. gondii* was the cause of abortion in the present study could not be determined because the brains were not examined histologically. Stalheim et al. (1980) mentioned in their paper that their colleagues had isolated *T. gondii* from the stomach contents of an aborted fetus in 1975, before the discovery of *N. caninum*. Their isolate of *T. gondii* was obtained by blind passages for 6 mo in cell cultures inoculated with the stomach contents from 1 of 255 aborted bovine fetuses. Because this is the only isolation of a *T. gondii*–like parasite (including *Neospora*) ever made from stomach contents, the significance of the findings is unknown. Lack of isolation of *T. gondii* in any of the aborted fetuses from California (Conrad et al., 1993) also suggests that *T. gondii* is not an important bovine abortifacient. However, the results here described warrant further investigation of the potential of *T. gondii* as a bovine abortifacient agent.

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